Langerhans Cells Favor Skin Flora Tolerance through Limited Presentation of Bacterial Antigens and Induction of Regulatory T Cells

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The mechanisms preventing detrimental T-cell responses against commensal skin bacteria remain elusive. Using monocyte-derived and skin-derived dendritic cells (DCs), we demonstrate that epidermal Langerhans cells (LCs), the DCs in the most superficial layer of the skin, have a poor capacity to internalize bacteria because of low expression of FcγRIIa. Furthermore, LCs show deficiency in processing and major histocompatibility complex II (MHC-II)-restricted presentation of bacterial antigens, as a result of a decreased expression of molecules involved in these functionalities. The reduced capacity to take up, process, and present bacterial antigens cannot be restored by LC activation by ectopically expressed Toll-like receptors or by cytokines. Consequently, bacteria-primed LCs poorly restimulate antibacterial memory $CD4^+$ T cells and inefficiently induce bacteria-specific effector $CD4^+$ T cells from naive T cells; however, they initiate the development of regulatory $Foxp3^+CD4^+$ T cells, which are able to suppress the proliferation of autologous bystander T cells specific for the same bacteria. In contrast, dermal DCs that reside in the deeper dermal layer of the skin efficiently present bacterial antigens and provoke robust antibacterial naive and memory $CD4^+$ T-cell responses. In conclusion, LCs form a unique DC subset that is adapted at multiple levels for the maintenance of tolerance to bacterial skin flora.

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INTRODUCTION

An important function of dendritic cells (DCs) is induction of antigen (Ag)-specific T-cell responses to control microbial infections (Banchereau and Steinman, 1998; Kapsenberg, 2003). Upon detection of microbes, DCs upregulate major histocompatibility complex (MHC) and costimulatory molecules, produce inflammatory cytokines, and migrate to draining lymph nodes to activate T cells. $CD4^+$ T-cell activation depends on the capacity of DCs to present exogenous Ags on MHC-II. To present Ags to activate $CD4^+$ T cells, DCs internalize and process microorganisms. During the process of internalization, microorganisms are encapsulated by the plasma membrane forming an intracellular vacuole known as the phagosome. The phagosome undergoes a series of regulated fusion events with endocytic organelles, first with endosomes and subsequently with lysosomes, modifying their composition (Trombetta and Mellman, 2005; Burgdorf and Kurts, 2008). During this process, internalized material is degraded into small fragments and loaded onto MHC-II. The type and quality of T-cell responses are additionally determined by costimulatory and polarizing signals (Banchereau and Steinman, 1998; Kapsenberg, 2003). Memory $CD4^+$ T cells do not require extensive costimulation and mainly depend on Ag presentation for optimal activation, whereas Ag presentation to naive $CD4^+$ T cells in absence of costimulation may lead to T-cell anergy or tolerance (Banchereau and Steinman, 1998; Steinman *et al.*, 2003; Iwasaki and Medzhitov, 2004).

In human skin, initiation of T-cell responses mainly depends on two different DC subsets: Langerhans cells (LCs) (Rowden *et al.*, 1977; Teunissen *et al.*, 2012), which form a three-dimensional network in the epidermis, and interstitial

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Abbreviations: Ag, antigen; CLSM, confocal laser scanning microscopy; DC, dendritic cell; DDC, dermal DC; E. coli, Escherichia coli; EEA-1, Early Endosome Antigen 1 protein; EM, electron microscopy; FcR, Fcγ receptor; LAMP-1, lysosomal-associated membrane protein 1; LC, Langerhans cell; MHC, major histocompatibility complex; MFI, mean fluorescence intensity; S. aureus, Staphylococcus aureus; S. epidermidis, Staphylococcus epidermidis; TLR, Toll-like receptor

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dermal DCs (DDCs), which are solely found in the dermal layer of the skin (Rowden *et al.*, 1977; Nestle *et al.*, 1998; Teunissen *et al.*, 2012). LCs do not express cell surface Toll-like receptor TLR2, TLR4, and TLR5, and thus resulting in a limited responsiveness of LCs to extracellular bacteria in terms of upregulation of costimulatory molecules and production of inflammatory cytokines. In marked contrast, DDCs do express these TLRs and vigorously respond to bacteria (Takeuchi *et al.*, 2003; Flacher *et al.*, 2006; van der Aar *et al.*, 2007). It is attractive to hypothesize that because LCs reside in close contact with the bacterial skin flora, they have a limited function in the initiation of antibacterial immunity to prevent excessive inflammation, whereas DDCs are specialized in the induction of antibacterial immunity. Furthermore, the question remains whether, and by what mechanisms, LCs prevent reactivation of bacteria-specific memory T cells that are present in the skin. We show that LCs have a very low capacity to internalize and process bacteria, resulting in a limited MHC-II-mediated presentation of bacterial Ags. In addition, bacteria-primed LCs can drive the development of Foxp3$^+$ regulatory CD4$^+$ T cells. Thus, LCs possess several intrinsic properties that prevent induction of strong, probably unwanted, antibacterial CD4$^+$ T-cell responses. We propose that these unique properties of LCs render the epidermis into a selective immune-privileged site to accommodate interaction with the commensal bacterial skin flora, whereas the dermis, with DDCs as bacteria-recognizing sentinels, forms the true immunological barrier for the skin against penetrating bacteria.

RESULTS

LCs are weak activators of bacteria-specific CD4$^+$ T cells and induce bacteria-specific Foxp3$^+$ T cells

To get insight into the role of LCs and DDCs in the induction of antibacterial T-cell responses, we compared their ability to stimulate naive autologous CD4$^+$ T cells specific for Gram-positive *S. aureus* or Gram-negative *E. coli*. Clearly, the induction of naive T-cell proliferation by LCs was less compared with the proliferation induced by DDCs at different DC:T cell ratios (Figure 1a and Supplementary Figure S1a online). This was in line with the expectations as bacteria induce high levels of costimulatory and MHC-II molecule expression by DDCs but not by LCs (Takeuchi *et al.*, 2003; Flacher *et al.*, 2006; van der Aar *et al.*, 2007). To obtain full maturation of LCs, we added IL-1β/TNF-α (MF) together with *S. aureus* (Supplementary Figure S1b online). This increased their capacity to induce bacteria-specific T-cell proliferation, however, not to the same extent as bacteria-loaded DDCs (Figure 1b), demonstrating that additional factors, such as the amount of presented bacterial Ags, have a role in the inferior capacity of LCs to prime antibacterial CD4$^+$ T cells. Remarkably, the T cells activated with *S. aureus*-primed or *E. coli*-primed LCs contained a high percentage of Foxp3$^+$CD127^{neg} cells, a phenotype associated with regulatory T cells (Tregs), whereas this population was almost absent when DDCs were used (Figure 1c and Supplementary Figure S1c online). Addition of MF during bacteria loading of LCs abrogated the formation of Foxp3$^+$ T cells (Figure 1c and Supplementary Figure S1c online).

To investigate whether the T cells primed by *S. aureus*-primed LCs indeed acquired regulatory capacities, we tested their ability to suppress the proliferation of activated bystander CD4$^+$ T cells. Indeed, the T cells promoted by *S. aureus*-primed LCs were able to suppress the proliferation of autologous bystander T cells, whereas the T cells promoted by *S. aureus*-primed DDCs did not suppress the proliferation (Figure 1d), confirming that the Foxp3$^+$ T cells induced by *S. aureus*-primed LCs are indeed regulatory T cells. In line with the absence of Foxp3$^+$ T cells, the presence of MF abrogated the suppressive capacity of the LC-induced T cells. Moreover, inhibition of TGF-β signaling by SMAD3 inhibitor, almost completely abrogated development of Foxp3$^+$ T cells (Figure 1e), showing an essential role for TGF-β in the induction of these Tregs.

In contrast to naive T cells, effector T cells do not require extensive costimulation and are mainly dependent on Ag presentation. We investigated to what extent bacteria-loaded LCs and DDCs induce the proliferation of autologous bacteria-specific effector CD4$^+$ T cells (Supplementary Figure S1d online). *S. aureus*-loaded LCs consistently induced significantly lower proliferation of *S. aureus*-specific T-cell lines compared with DDCs at all bacteria:DC ratios and DC:T cell ratios tested (Figure 1f and g). The same results were obtained with *E. coli* (Supplementary Figure S1e and f online). Addition of MF did not enhance the proliferation of bacteria-specific T cells, confirming that lack of costimulation did not have a role. Collectively, these data indicate that LCs are not only inefficient in priming bacteria-specific naive T cells but also in reactivating memory T cells.

LCs inefficiently internalize bacteria because of low CD32 expression

To investigate whether the low efficiency of LCs to activate T cells is the result of a limited capacity to internalize particulate Ag, we incubated LCs and DDCs with GFP-expressing bacteria and quantified internalization by FACS (Suplementary Figure S2a). LCs internalized significantly lower amounts of *S. aureus* or *E. coli* compared with DDCs at all bacteria:DC ratios tested. Not only was the percentage of LCs that internalized bacteria lower than that of DDCs but also the mean amount of bacteria taken up per cell was 3–6 times less for LCs (Figure 2a and Supplementary Figure S2b and c online). To verify the *in vivo* relevance of these findings, we investigated bacterial uptake by skin-derived LCs and DDCs and again observed the same differences (Figure 2b and Supplementary Figure S2d and e online). The differences in the bacterial uptake were confirmed by confocal laser scanning microscopy (CLSM), electron microscopy (EM), and multispectral imaging (Supplementary Figure S2f–h online). No internalization was observed at 4 °C (data not shown), indicating that active uptake of the bacteria was required for internalization. Moreover, after a longer time period LCs did not reach the same level of uptake of *S. aureus* as DDCs (Supplementary Figure S2i and j online), showing that LCs did not simply require more time to internalize the same number of bacteria as DDCs.

EM analysis revealed that LCs regularly (~50% of the cases) formed a phagocytic cup to internalize bacteria, whereas

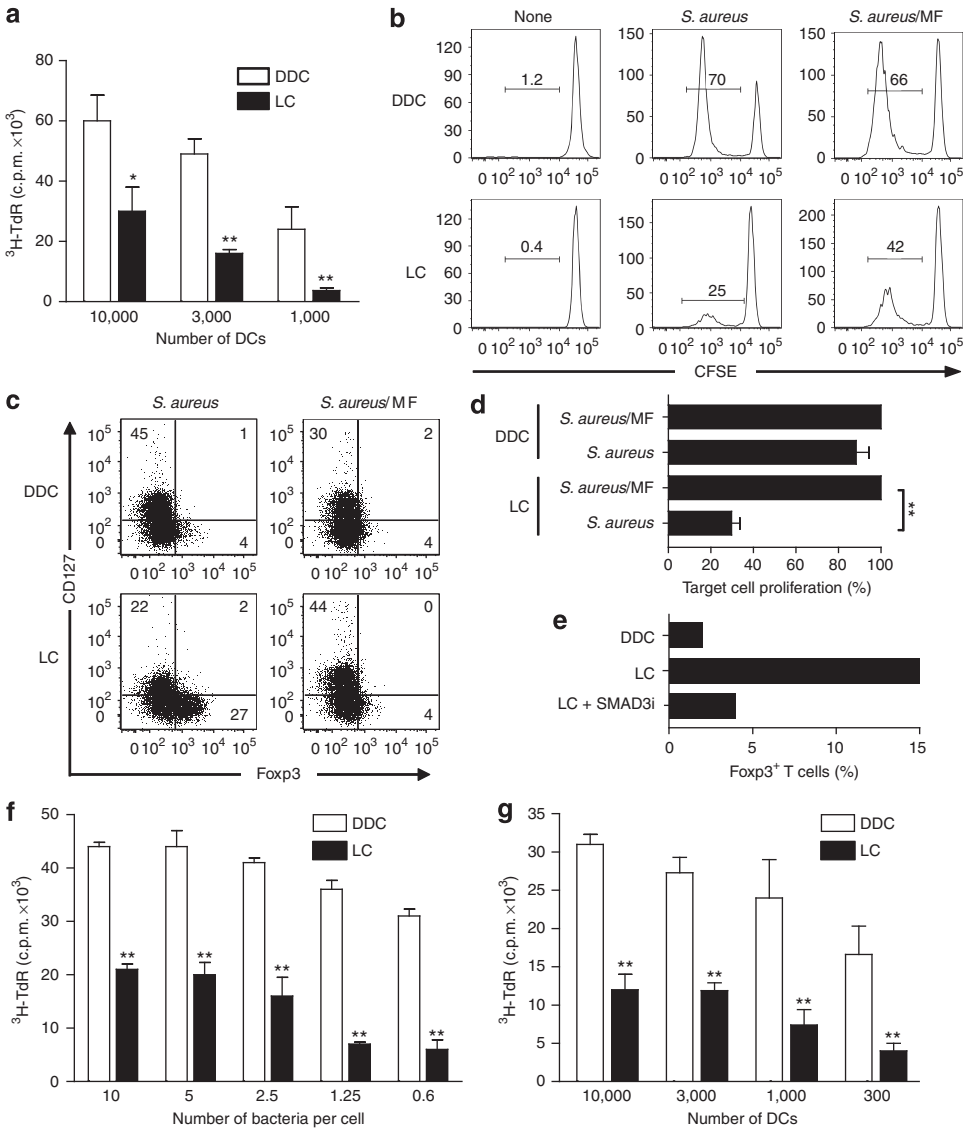

Figure 1. Limited activation of Langerhans cells (LCs) by bacteria results in impaired activation of bacteria-specific T cells. (**a**) Proliferation of naive CD4$^+$ T cells cocultured with *S. aureus*-loaded *in vitro*–generated dermal dendritic cells (DDCs) and LCs. The mean ± SD of four independent experiments is shown (*P<0.05; **P<0.01). (**b**) Representative graphs (n=5) for the proliferation of naive CD4$^+$ T cells after coculture with *S. aureus*-loaded DDCs and LCs in the absence or presence of IL-1β/TNF-α (MF). (**c**) CD127 and intracellular Foxp3 staining on T cells primed with *S. aureus*-loaded DDCs or LCs in the absence and presence MF. Data are representative for three independent experiments. (**d**) The proliferation of activated bystander CD4$^+$ T cells (target cells) in the presence of T cells induced by *S. aureus*– or *S. aureus*/MF–primed DDCs or LCs. The proliferation of target T cells in the presence of T cells stimulated with *S. aureus*/MF–DCs was set at 100%. Data show the mean ± SD of three independent experiments (**P<0.01). (**e**) Percentage of Foxp3$^+$ CD4$^+$ T cells induced by *S. aureus*-loaded LCs in the absence (solvent control DMSO) or presence of SMAD3 inhibitor (representative for three independent experiments). (**f**, **g**) The proliferation of bacteria-specific memory CD4$^+$ T cells upon activation by *S. aureus*-loaded DDCs and LCs. Data show the mean ± SD of four independent experiments (**P<0.01).

DDCs did not (Figure 3a), suggesting that LCs and DDCs use different uptake mechanisms, but treatment with various inhibitors showed that bacteria were internalized through a clathrin-independent and caveolin-independent mechanism requiring actin polymerization by both DC subsets (Supplementary Figure S3a–c online). Lack of inhibition by mannan showed that C-type lectins were also not involved (Supplementary Figure S3d online). However, internalization of bacteria was substantially reduced when human serum was absent (Figure 3b), suggesting an important role for complement receptors and/or FcRs. A major contribution by complement was ruled out as heat inactivation did not affect uptake (Supplementary Figure S3e online). Opsonization of *S. aureus* with purified human Ig enhanced bacterial internalization significantly, and blocking of FcRs reduced the bacterial uptake by both DC subtypes to the level of serum-free conditions (Figure 3b). Bacterial uptake by skin-derived and *in vitro*–generated LCs and DDCs was significantly reduced by blocking CD32 but not by blocking CD16 or CD64 (Figure 3c and d). FACS analysis revealed a markedly lower expression of FcRs by LCs than by DDCs (Figure 3e and f). Collectively, these data indicate that the differences in bacterial uptake by

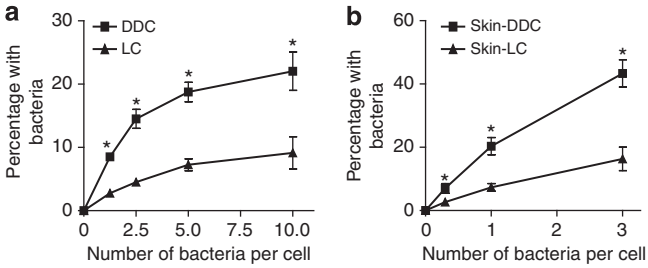

Figure 2. Langerhans cells (LCs) are inefficient in internalizing bacteria compared with dermal dendritic cells (DDCs). *In vitro*–generated (**a**) or skin-derived (**b**) DDCs and LCs were incubated for 1 hour with the indicated number of GFP-expressing *S. aureus*. Depicted is the percentage of cells that internalized *S. aureus*. Data show the mean ± SD from 10 (**a**) or 3 (**b**) independent experiments, $*P<0.05$.

LCs and DDCs can be attributed to different expression levels of CD32.

LCs have a low expression of molecules involved in MHC-II processing and presentation and are inefficient at processing bacterial Ags

To determine to what extent the inefficient bacterial uptake by LCs contributed to low activation of bacteria-specific T cells, we selected LCs and DDCs with equal bacterial load and compared their capacity to activate Ag-specific $CD4^+$ T cells. Strikingly, although the differences between LCs and DDCs became smaller, the ability to stimulate bacteria-specific T-cell proliferation was still significantly lower for LCs (Figure 4a), showing that the limited internalization capacity of LCs is partly responsible for the low efficiency to activate bacteria-specific T cells. Efficient Ag presentation by DCs also depends on effective degradation of internalized bacterial cargo in endosomal compartments. We analyzed phagosome maturation by CLSM and immuno-EM and found that *S. aureus* initially colocalized with $EEA\text{-}1^+$ early endosomes and that in both LCs and DDCs, these bacteria-containing early endosomes matured into late endosomes/lysosomes, as indicated by the acquirement of late endosomal/lysosomal markers LAMP-1 and HLA-DM (Figure 4b). However, the number of bacteria colocalizing with LAMP was much less in LCs than in DDCs. Moreover, immuno-EM also revealed that CD63 acquisition of phagosomes was evidently less in LCs than in DDCs (arrows, Figure 4c). Quantification of colocalization of *S. aureus* with $EEA\text{-}1^+$ and $LAMP^+$ endosomes by multispectral imaging FACS (Supplementary Figure S4 online) demonstrated that the percentage of internalized bacteria that colocalized with LAMP was twice as low in LCs compared with DDCs, and in LCs a high percentage of the internalized bacteria still colocalized with EEA-1 (Figure 4d). Collectively, these data indicate that after bacterial uptake, phagosome maturation into late endosomal/lysosomal compartments is slower in LCs than in DDCs.

The capacity of LCs to present bacterial Ags is not enhanced by TLR signaling

Phagosome maturation and the capacity to present Ags through the MHC-II pathway have been linked to TLR triggering by the internalized material (Blander and Medzhitov, 2006). As LCs do not express bacterial sensors TLR2, TLR4, and TLR5, we examined whether the inefficient presentation of bacterial Ags by LCs could be overcome by triggering TLR signaling pathways TRIF and MyD88 in an alternative manner, using TLR3 and TLR7, respectively, as LCs express these TLRs. LCs also express TLR1 and TLR6, but triggering through these TLRs would not be appropriate, as both TLR1 and TLR6 have to form a heterodimer with TLR2 to become functional, and LCs lack TLR2. The addition of TLR3 or TLR7 ligand during bacterial uptake only slightly enhanced bacterial uptake (Figure 5a) and did not affect their induction of bacteria-specific T-cell proliferation (Figure 5b). To address the possibility that TLR signaling must be triggered specifically at the site of phagosome formation, that is, involving local cell surface TLRs, we generated $TLR2^+$ LCs using mRNA transfection. These transfected LCs responded to TLR2-ligand Pam3CSK by producing comparable levels of IL-6 as DDCs (Supplementary Figure S5 online) showing functionality of the ectopically expressed TLR2. However, the TLR2 expression by LCs did neither enhance the capacity to internalize bacteria (Figure 5c) nor the capacity to induce T-cell proliferation (Figure 5d), showing that the low efficiency of LCs to present bacterial Ags is not because of the absence of TLRs that recognize bacteria.

LCs have a low expression of molecules involved in MHC-II processing and presentation

The observation that phagosomal maturation is slower in LCs than in DDCs indicates that LCs differ in their capacity to process Ags and to load Ags onto MHC-II molecules. Microarray analysis revealed a markedly low expression of many genes encoding proteins that are associated with Ag processing in the MHC-II pathway by LCs (Figure 6a). LCs also showed low expression of genes involved in the regulation of Ag loading onto MHC-II molecules such as HLA-DM and HLA-DO. No significant differences were found in mRNA expression of Ii and master transcriptional regulator of class II genes CIITA. These findings were confirmed by quantitative PCR and FACS on skin-derived and *in vitro*–generated LCs and DDCs (Supplementary Figure S6a–c online). The higher expression of RAB14 in LCs (Figure 6a) is interesting, as this small GTPase attenuates the fusion of phagosomes with lysosomes (Kyei *et al.*, 2006) and consequently may contribute to the low Ag processing in LCs. Although not significant, we found in LCs a higher expression of MARCH1, which negatively regulates Ag presentation by ubiquitinating MHC class II and reducing its surface expression. (de Gassart *et al.*, 2008).

Incubation with OVA bound to a self-quenched fluorochrome confirmed that LCs are considerably less efficient compared with DDCs in processing Ags, as indicated by the release of fluorescent decomposition products (Figure 6b). To investigate whether LCs and DDCs show a differential capacity for MHC-II Ag loading, we compared their efficacy to exchange CLIP for antigenic peptides upon incubation with *S. aureus*. For this, we analyzed the relative expression of stable MHC-II/Ag complexes using mAb 16.23, specifically recognizing HLA-DR3/Ag complexes (Johnson *et al.*, 1982) and the

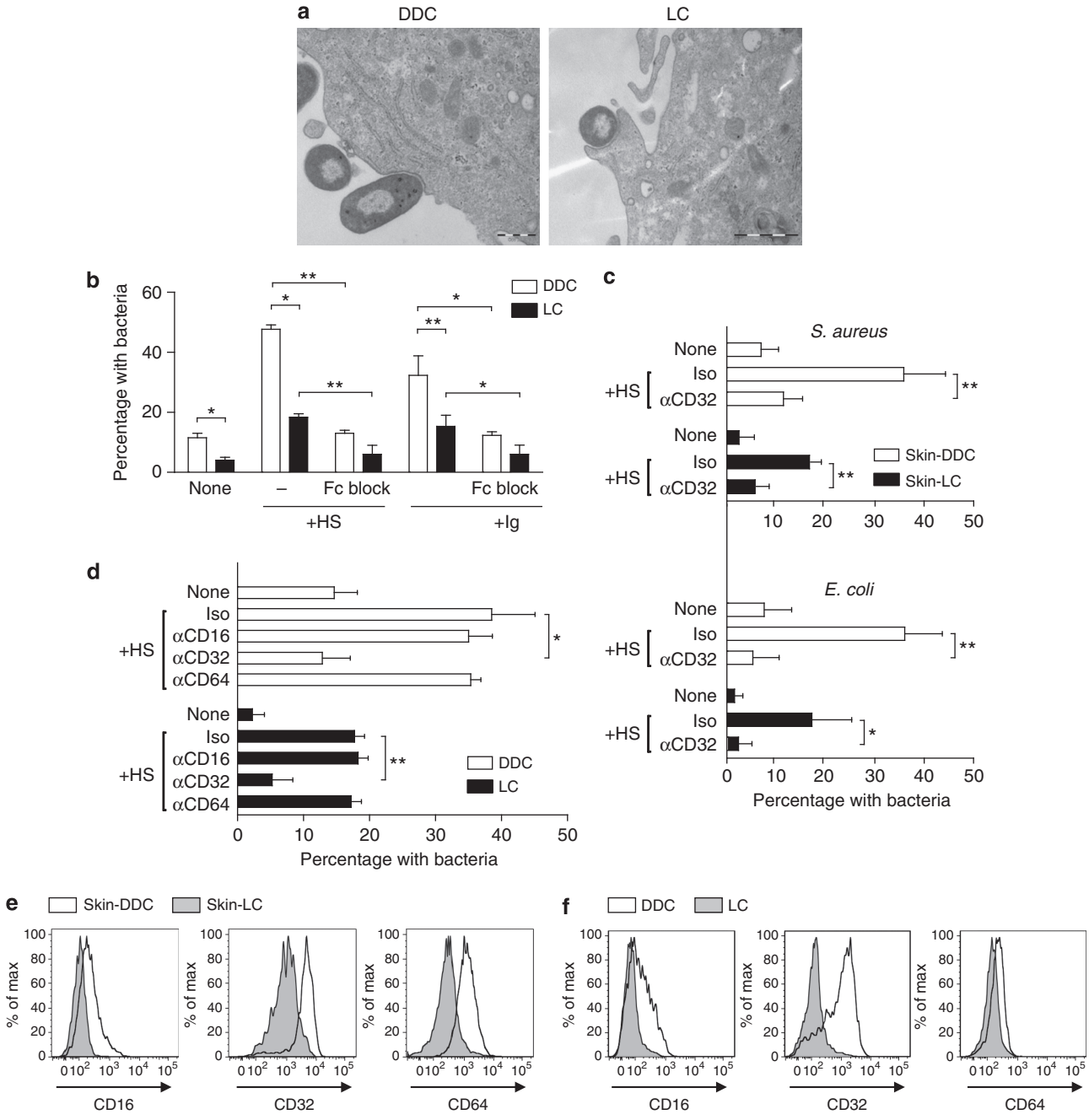

Figure 3. Low bacterial uptake of Langerhans cells (LCs) is attributed to low FcγRIIa expression. (**a**) Representative electron microscopy (EM) picture of dermal dendritic cells (DDCs) and LCs during internalization of *S. aureus*. (**b**) FACS analysis of uptake of *S. aureus*–GFP by *in vitro*–generated DDCs and LCs after opsonization with HS or purified human Ig in the absence (–) or presence of antibodies for blocking total FcR. (**c**) Uptake of *S. aureus* or *E. coli* by skin-derived DDCs and LCs in the absence (none) or presence of HS and blocking CD32 antibodies or IgG2b isotype control (iso). (**d**) Uptake of *S. aureus* by *in vitro*–generated DDCs and LCs in the absence (none) or presence of HS and blocking CD16, CD32, CD64 antibodies, or IgG1 and IgG2b isotype control antibodies (iso). (**e**) CD16, CD32, and CD64 expression on skin-derived DDCs and LCs or (**f**) *in vitro*–generated DDCs and LCs. Data show one representative (**a**, **e**, **f**) or the mean ± SD from three independent experiments (**b**–**d**, $*P<0.05$; $**P<0.01$).

mAb CerCLIP.1 that recognizes MHC-II/CLIP complexes. FACS showed that *S. aureus* DDCs upregulated not only total HLA-DR but also the fraction of stable HLA-DR3/Ag complexes with a decrease in the relative expression of HLA-DR/CLIP. In contrast, incubation of LCs with *S. aureus* stimulated MHC-II export to some extent, but the amount of HLA-DR3/Ag complexes did not increase (Figure 6c). This indicates that the increase of MHC-II export to the plasma membrane is accompanied by efficient exchange of CLIP for antigenic peptides in DDCs and not in LCs. These observations are in line with the low expression of HLA-DM in LCs. In summary, these data demonstrate that LCs are less efficient compared with DDCs in Ag processing and Ag loading onto MHC-II molecules.

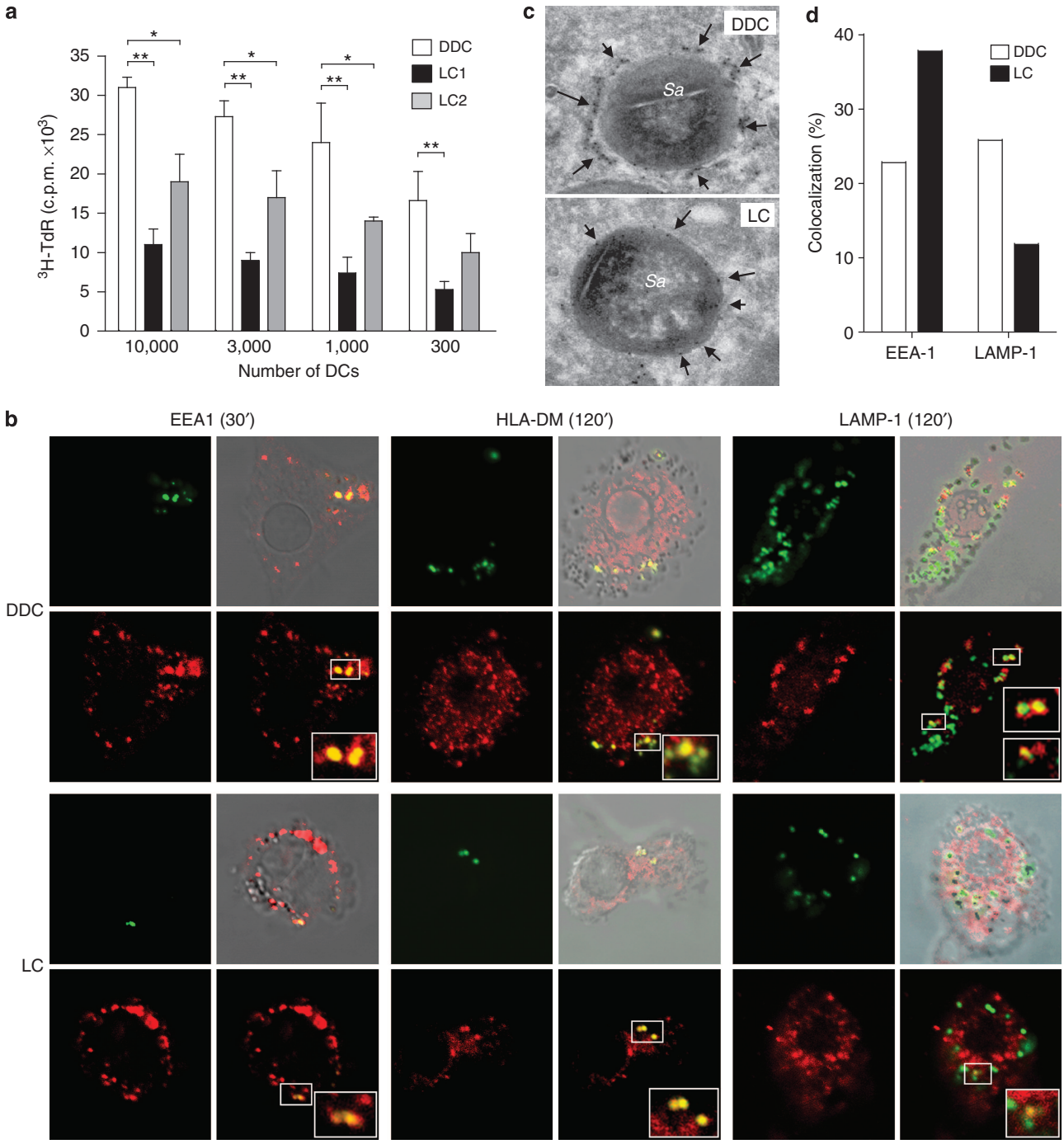

Figure 4. Langerhans cells (LCs) are inefficient in processing bacterial antigens. (**a**) Dermal dendritic cells (DDCs) and LCs with normal bacterial load (LC1) and LCs with an equal bacterial load as the DDCs (LC2, selected by FACS) were cocultured with 40,000 bacteria-specific CD4$^+$ T cells. T-cell proliferation was measured by [^{3}H]-thymidine incorporation. Data show the mean ± SD of four independent experiments (*$P<0.05$; **$P<0.01$). (**b**) DC subsets were incubated for 0.5–2 hours with S. *aureus*–GFP and subsequently stained for endosomal markers. Colocalization of bacteria with endosomes was analyzed by confocal laser scanning microscopy. Figures show representative serial Z-stack images for colocalization of bacteria in DDCs and LCs with Early Endosome Antigen 1 protein (EEA-1) (30 minutes), HLA-DM (120 minutes), and lysosomal-associated membrane protein 1 (LAMP-1) (120 minutes). Colocalization appears yellow, and insets show boxed areas with enlarged colocalization and correlating bright-field images. (**c**) Analysis of internalization of *S. aureus* after 2 hours by immuno-EM. Arrows indicate one representative bacteria-containing phagosome stained with CD63-gold immuno-label. (**d**) Quantification of colocalization of bacteria with EEA-1 and LAMP-1 by multispectral imaging FACS 1.5 hours after incubation. The amount of bacteria colocalizing with EEA-1$^+$ or LAMP-1$^+$ endosomes was calculated as a percentage of the total amount of internalized bacteria. Data are representative of three independent experiments.

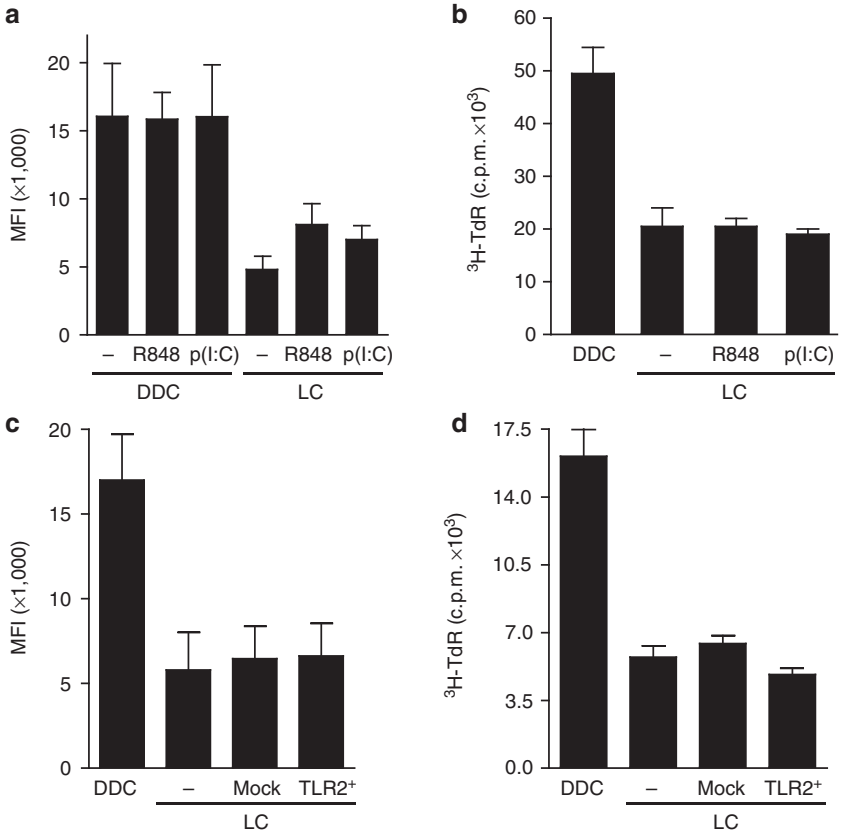

Figure 5. The capacity of Langerhans cells (LCs) to present bacterial antigens is not enhanced by Toll-like receptor (TLR) signaling. (**a**) Internalization of *S. aureus*–GFP by *in vitro*–generated dermal dendritic cells (DDCs) and LCs 1 hour after coincubation in the absence or presence of TLR ligands R848 or p(I:C), showing the mean fluorescence intensity (MFI) as measurement of the numbers of internalized bacteria by LCs and DDCs. (**b**) The proliferation of bacteria-specific CD4$^+$ T cells after incubation with DDCs and LCs loaded with *S. aureus* in the absence or presence of R848 or p(I:C). (**c**) Internalization of *S. aureus* and (**d**) induction of bacteria-specific CD4$^+$ T-cell proliferation by DDCs, and untreated (–), mock-treated, or TLR2$^+$ LCs. Data in **a**, **b**, and (**c**, **d**) are the mean ± SD of three, four, and five independent experiments, respectively.

DISCUSSION

This study unequivocally shows that internalization, degradation, and MHC-II loading of bacterial Ags are functions that are poorly performed by LCs, resulting in a low activation of bacteria-specific T cells. A low capacity to internalize bacteria by LCs is supported by a study showing that only a minority of freshly isolated human LCs internalized Gram-negative *Borrelia burgdorferi*, whereas freshly isolated DDCs eagerly internalized these bacteria (Filgueira *et al.*, 1996). Here, we show impaired internalization of not only Gram-negative *E. coli* but also Gram-positive bacteria *S. aureus*.

The low capacity of LCs to internalize and process bacteria was attributed to several intrinsic properties, such as impaired FcγR expression and low expression of molecules related to the MHC-II-processing machinery. The low expression of MHC-II-processing-related genes by LCs is in line with a previous report (Klechevsky *et al.*, 2009), which did not address functional consequences. Highly purified, freshly isolated authentic human epidermal LCs selectively lack bacterial sensors TLR2, TLR4, and TLR5 rendering these cells unable to respond efficiently to bacteria (van der Aar *et al.*, 2007). However, some reports show responsiveness of LCs to TLR2 and TLR4 agonists (Peiser *et al.*, 2008; Aliahmadi *et al.*, 2009; Duraisingham *et al.*, 2009; Jurkin *et al.*, 2010; Peña-Cruz *et al.*, 2010). These contradictory observations may be explained by differences in laboratory protocols, purity of the LCs, or LC-model used (CD34 stem cell–derived, monocyte-derived, or epidermis crawl-out matured LCs), providing LC populations with variant TLR profiles and responsiveness to TLR triggering, not resembling those of bona fide epidermis-derived immature LCs. Although a link between adequate TLR triggering and efficiency to process and present Ags has previously been reported (Blander and Medzhitov, 2006), the limited uptake of bacteria and Ag presentation by LCs could not be enhanced by introducing functional TLR2 triggering. As a consequence of the limited presentation of bacterial Ags, LCs not only have a limited capacity to activate naive T cells but also, in contrast to DDCs, provoke weak reactivation of memory CD4$^+$ T cells, which do not require high levels of costimulation. In addition, the presence of inflammatory cytokines IL-1β and TNF-α could not enhance the stimulation of bacteria-specific memory T cells, suggesting that *in situ* LCs are not easily provoked by activated surrounding keratinocytes to induce antibacterial T-cell immunity.

Interestingly, bacteria-primed LCs, and not bacteria-primed DDCs, induced a high percentage of bacteria-specific Foxp3$^+$

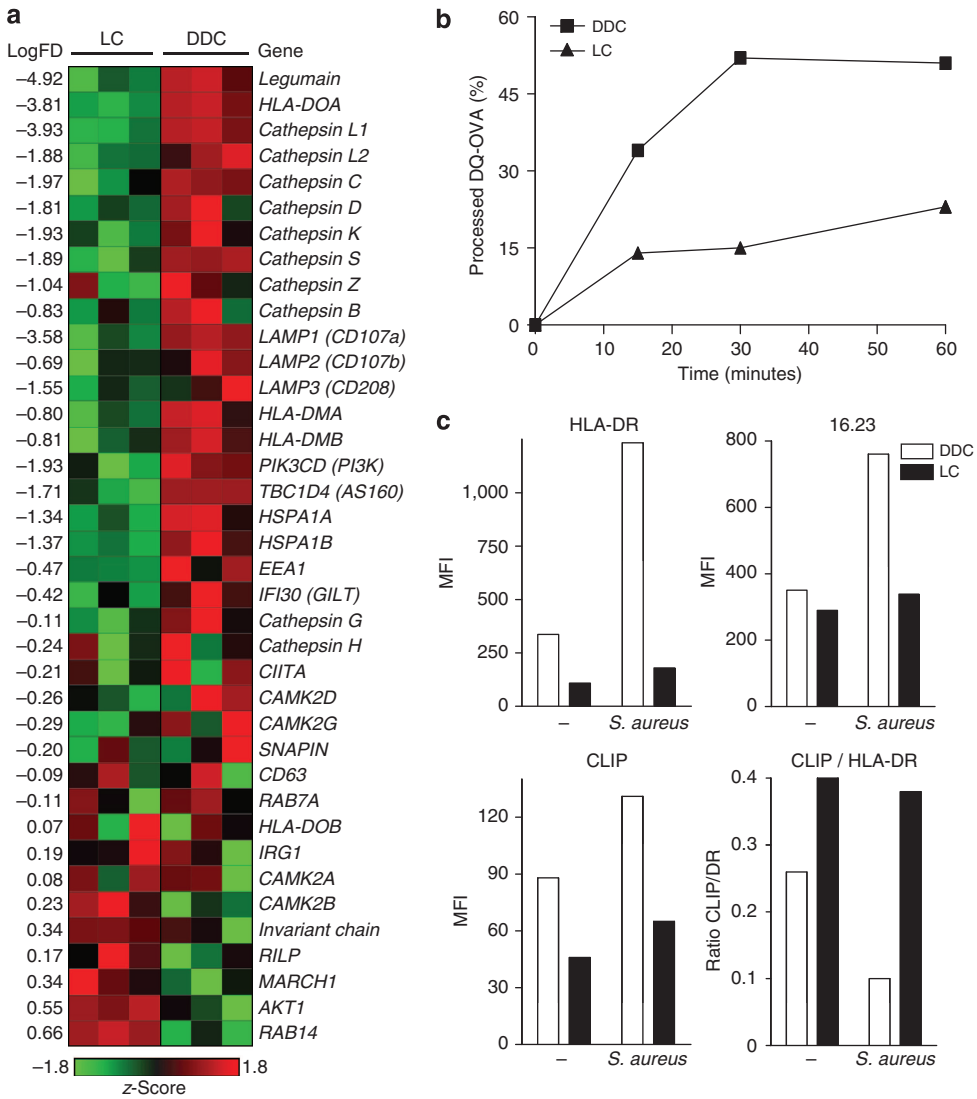

Figure 6. Langerhans cells (LCs) have a low expression of molecules associated with MHC-II antigen processing and antigen loading. (**a**) Columns in the heat map represent relative amounts of mRNAs of molecules associated with MHC-II-processing pathways and expressed by *in vitro*–generated dermal dendritic cells (DDCs) and LCs from three different donors as determined by gene array analysis. Data are shown as *z*-scores, and fold differences are indicated on the left side. (**b**) DQ-OVA processing by DDCs and LCs at various time points after internalization (horizontal axis), indicated by formation of ''nonquenched'' DQ-OVA degradation product. Results are normalized to the uptake of DQ-OVA and are shown as % of the total internalized amount. (**c**) FACS analysis of HLA-DR, HLA-DR3 stably bound to antigens (16.23), and CLIP on DDCs and LCs after 16-hour culture with or without *S. aureus*. Data (**b**, **c**) show one representative out of three independent experiments. MFI, mean fluorescence intensity.

T cells, enforcing the concept that LCs have a role in maintaining tolerance to the bacterial skin flora (Campbell and Ziegler, 2007; Vignali *et al.*, 2008). Indeed, suboptimal DC activation (Hawiger *et al.*, 2001; Menges *et al.*, 2002; Steinman *et al.*, 2003) and very low doses of Ags (Turner *et al.*, 2009), or a combination of these factors (Kretschmer *et al.*, 2005), have previously been linked to the induction of Tregs. Addition of inflammatory cytokines that induce maturation of LCs abrogated the development of $Foxp3^+$ T cells, underscoring the concept that immature or semimature DCs can induce Ag-specific tolerance. This combination of lack of costimulation and inflammatory cytokines, due to the selective absence of cell surface TLRs, and the inefficient presentation of bacterial Ags by LCs appears deliberately aimed to prevent them from activating antibacterial effector T cells and renders them prone to induce bacteria-specific Tregs.

Considering that epidermal LCs are the only DC subset in the outermost layer of our body and that they are the first immune cells to sense danger of invading pathogens, our results may be quite unexpected. However, the limited capacity of LCs to induce potent antibacterial T-cell immunity may prevent harmful adaptive immune responses to the commensal skin flora. We propose that the epidermis is a site of immune tolerance to accommodate symbiotic interaction with commensal bacteria, whereas bacteria penetrating the dermal layer will readily activate DDCs. In line with our concept, keratinocytes, the major constituent of the epidermis,

need very high amounts of bacterial compounds to become activated (Lebre *et al.*, 2007).

TGF-β seems to have a dominant regulatory role in the maintenance of tolerance to the microbial skin flora. This cytokine is locally produced in the normal epidermis and critically determines the functional properties of LCs, including their selective TLR expression (Borkowski *et al.*, 1996; Thomas *et al.*, 2001; van der Aar *et al.*, 2007). In addition, this study shows that low bacterial uptake capacity and expression of molecules involved in the MHC-II Ag processing and presentation pathway, which all underlie LCs' inefficiency in mounting antibacterial T-cell responses, are under the influence of TGF-β. Furthermore, TGF-β is a key cytokine for the induction of Foxp3$^+$ Tregs (Vignali *et al.*, 2008). Indeed, induction of Foxp3$^+$ Tregs was abrogated by an inhibitor of SMAD3, supporting a role for LC-derived TGF-β. The *in vivo* relevance of the induction of Tregs by LCs to maintain tolerance for the microbial skin flora remains to be established. In other environments that are in close contact with commensal microbes, DCs are also important in maintaining tolerance by inducing Tregs. For example, in the gut, CD103$^+$ DCs use endogenous TGF-β and retinoic acid to induce Foxp3$^+$ Tregs (Coombes *et al.*, 2007). Moreover, Allam *et al.* (2008) reported that oral mucosal LCs, which in contrast to epidermal LCs do express TLR4, induce Foxp3$^+$ T cells in response to TLR4 ligation, by upregulating TGF-β and IL-10, which may be important for maintaining tolerance for the oral microbial flora.

In conclusion, this study presents evidence that DDCs are the main Ag-presenting cells in the skin for antibacterial immune responses and that LCs, to maintain a state of tolerance for the commensal skin flora, are adapted to avoid harmful constant induction and reactivation of antibacterial T cells.

MATERIALS AND METHODS

Preparation of LCs and DDCs

LCs and DDCs were generated from monocytes or purified from human skin as described (van der Aar *et al.*, 2007). Donation of skin by healthy controls followed approval by the ethical committee of the Academic Medical Center (Amsterdam, The Netherlands). This study was conducted according to the Declaration of Helsinki and subjects gave written informed consent. LCs were electroporated on d7 with mRNA as described (Michiels *et al.*, 2005).

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Analysis performed as described (de Jong *et al.*, 2002) using αMHC-II-PERCP, αHLA-DM-PE, αLAMP-1-APC, αEEA-1, αCLIP-FITC, αCD86-APC, and αCD83-PE (BD Biosciences, San Diego, CA), αCD16-FITC, αCD64 (Sanquin Research, Amsterdam, The Netherlands), αCD32-PE (Immunotech, Marseille, France), αDR3β mAb 16.23 (Johnson *et al.*, 1982), GαM-Alexa-405 (Molecular Probes, Invitrogen, Karlsruhe, Germany), GαM-PE and GαM-Cy3 (both Jackson Immunoresearch Laboratories, West Groove, PA).

Internalization and processing assays

DCs were cocultured with GFP-expressing *S. aureus* RN4220 and *E. coli* strain DH5α (Rooijakkers *et al.*, 2005) (five bacteria per cell unless stated otherwise) in IMDM (Life Technologies, Paisley, UK) containing 5% human serum (BioWhittaker, Walkersville, MD). Uptake was stopped by cold phosphate-buffered saline and analyzed by FACS. Antibodies against LTA (QED Bioscience, San Diego, CA) or *E. coli* (AbD Serotec, Kidlington, UK) were used to detect extracellular bacteria. When indicated, bacteria were preincubated for 30 minutes with human Ig (IVIG, Sanquin), FcR block (Miltenyi Biotec, Bergisch Gladbach, Germany), blocking antibodies (10 μg ml^{-1}) against CD16 (3G8, BD), CD32 (IV.3, Stemcell Technologies, Grenoble, France), or CD64 (10.1, BD), or with isotype controls IgG1 (MOPC-21, BD) or IgG2b (GL198, Sanquin), Mannan (500 μg ml^{-1}), Cytochalasin D, MDC (50 μM), and nystatin (2.5–10 μM, Sigma-Aldrich, St Louis, MO). For TLR triggering p(I:C) (Sigma-Aldrich) and R848 (InvivoGen, San Diego, CA) were used. For CLSM, DCs were stained with αLAMP-1-Alexa-647 (Biolegend, San Diego, CA), αEEA-1, αHLA-DM, and αCD11c (BD Pharmingen, San Diego, CA). Quantitative analyses of colocalization with endosomes were performed by multispectral imaging FACS with the ImageStream system (Amnis Corporation, Seattle, WA) using the ratio of overlapping GFP and EEA-1$^+$ and LAMP-1$^+$ spots to the total number of GFP spots. To investigate Ag processing, DCs were incubated for 1 hour with self-quenching DQ-OVA (Molecular Probes). Digested fragments were measured using a red-light filter. EM was performed as described previously (Calafat *et al.*, 1997). Sections were incubated with αCD63 (Sanquin) followed by RαM antibody.

T-cell stimulation

DCs were incubated with bacteria (16 hours) in the absence or presence of IL-1β (25 ng ml^{-1}) and TNF-α (50 ng ml^{-1}) (PBH) or in the absence or presence of SMAD3 inhibitor (Sigma) or solvent control DMSO and cocultured with autologous CD45RA$^+$ CD45RO$^-$ naive CD4$^+$ T cells as described previously (de Jong *et al.*, 2002) or with autologous pathogen-specific T-cell lines (40×10^4). T-cell proliferation was measured by [^{3}H]-thymindine incorporation after 24, 48, or 72 hours. Simultaneously, proliferating T cells were FACS-sorted on day 5 and cultured in the presence of IL-2. Resting T cells were analyzed for CD127 (BD Pharmingen), intracellular Foxp3, and suppressive capacity as described previously (van der Aar *et al.*, 2011). Bacteria-specific T-cell lines were prepared as described (Kalinski *et al.*, 2003).

Quantitative PCR and microarray analysis

Gene expression of HLA-DRA, HLA-DMB, HLA-DOA, HLA-DOB, and CIITA was measured as described (Souwer *et al.*, 2009). Other primers used were: Ii (5′-CACCTGCTCCAGAATGCTG-3′, 5′-CAGTTCCAGTGACTCTTTCG-3′, 210 bp). Microarray was performed as described (Vroling *et al.*, 2008).

Statistics

Student *t*-tests were performed for paired measurements with GraphPad (version 4.00; GraphPad InStat, San Diego, CA). *P*-values <0.05 were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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